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The Insert Region of Rac1 Is Essential for Membrane Ruffling but Not Cellular Transformation

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The Rho family of Ras-related proteins, which includes Rac1, RhoA, and Cdc42, is distinguished from other members of the Ras superfamily of small GTPases in that its members possess additional sequences positioned between β-strand 5 and α-helix 4, designated the insert region. Previous studies have established the importance of an intact insert region for the transforming, but not actin cytoskeletal reorganization, activities of Cdc42 and RhoA. Similarly, the insert region was determined to be essential for Rac1-mediated mitogenesis. Additionally, an intact insert region was also determined to be required for the antiapoptotic activity of Rac1 as well as for Rac1 activation of reactive oxygen species and the NF-κB transcription factor. However, it has not been determined whether the insert region is important for Rac1-mediated growth transformation. In this study, we assessed the requirement for the insert region in Rac1 transformation and signaling in NIH 3T3 cells. Unexpectedly, we found that a mutant of constitutively activated Rac1 that lacked the insert region retained potent transforming activity. The insert region of Rac1 was dispensable for Rac1 stimulation of transcription from the cyclin D1 promoter and for activation of the c-Jun, NF-κB, and E2F-1 transcription factors but was essential for Rac1 induction of serum response factor activity. While an intact insert region was dispensable for inducing reactive oxygen species production in vivo, it was required for Rac1 induction of lamellipodia. When taken together, these results show that the insert region of Rac1 serves roles in regulating actin organization and cell growth that are distinct from those of the analogous regions of Cdc42 and RhoA and support its involvement in regulating specific downstream effector interactions.

Rac1 is a member of the Rho branch of the Ras superfamily of small GTPases. To date, 15 mammalian Rho family members have been identified, the best-characterized members being Rac1, RhoA, and Cdc42 (61, 66). Rho family proteins are binary switches that cycle between active GTP-bound and inactive GDP-bound states to regulate actin cytoskeletal organization, gene transcription, and cell growth. Their activity is positively regulated by Dbl family guanine nucleotide exchange factors (GEFs) which catalyze the exchange of GDP for GTP to promote formation of the GTP-complex protein. Downregulation is achieved by GTPase-activating proteins, which enhance the intrinsic GTP hydrolysis rate, and guanine nucleotide dissociation inhibitors, which interfere with GTP hydrolysis and inhibit GDP-GTP exchange, favoring the formation of the GDP-bound protein.

Rac1 plays a key regulatory role in various cellular processes, such as superoxide production, cell movement, proliferation, and apoptosis (61). Rac1 also controls gene expression by regulating the activity of transcription factors such as serum response factor (SRF) (19), NF-κB (39), E2F-1 (17), c-Jun, ATF-2, and Elk-1 (6). In addition, Rac1 promotes the reorganization of filamentous actin into membrane structures called lamellipodia or membrane ruffles, whereas RhoA and Cdc42 promote stress fiber and filopodium formation, respectively (18). Moreover, Rac1 is required for transformation induced by Ras (29, 46) and other oncoproteins (e.g., Vav, Abl, and Mas) (66). Finally, constitutive activation of Rac1 causes anchorage-independent growth, invasion, and metastasis (9, 27, 36).

In light of the diverse functions of Rac1, it is not surprising that a multitude of candidate effectors that bind preferentially to activated Rac1-GTP have been identified (3, 61, 66). Rac1 effectors include the PAK, MLK, and MEKK serine/threonine kinases, IQGAPs, POSH, PORI, and Par-6 (47). Presently, no one specific protein has been tied directly to the ability of Rac1 to transform cells and it is likely that multiple effectors will be involved in Rac1 regulation of cell proliferation.

An important approach for dissecting the contribution of specific effectors and effector-mediated signaling pathways in Rac function has been the analyses of mutants of Rac1 that are selectively impaired in a subset of effector functions. A majority of these studies have utilized missense mutations in the NH_2-terminal effector domain sequences of Rac1 that span residues 25 to 42 and include the core effector domain (residues 32 to 40) and the switch I and switch II domains, whose conformations are sensitive to the binding of GTP versus GDP. However, studies involving Ras-RhoA, Rac1-Cdc42, and Rac1-RhoA chimeras as well as missense mutations have identified additional NH_2- and COOH-terminal sequences that are important for Rac1 effector interactions (14, 32, 51, 62). Thus, multiple sequences of Rac1 are important for interaction with effectors.

Another region of Rac1 implicated in effector interaction has been the surface-exposed and dynamic insert region (residues 124 to 135). Unlike Ras and other members of the Ras superfamily, all Rho family GTPases share a variable length short amino acid insertion between β-strand 5 and α-helix 4, designated the insert region (60). Earlier studies initially de-

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termined that the insert region was dispensable for Rac1 and Cdc42 activation of JNK and PAK1 and for actin reorganization (24, 63). Mutants of Rac1 and Cdc42 lacking the insert region were still responsive to regulation by GTPase-activating proteins and GEFs and bound guanine nucleotides as well as their wild-type counterparts did (59, 63). However, recent reports indicated that the Rho family insert region is involved in effector interactions which are important for mediating cellular transformation. Mutation of the insert region was found to abolish Cdc42- and RhoA-mediated transformation of NIH 3T3 cells (64, 67). Similarly, deletion of the insert domain impaired Rac1-induced DNA synthesis in REF-52 cells (24). However, whether the insert region of Rac1 was also critical for transformation was not determined.

Other functions of Rac1 have also been found to require an intact insert domain. Rac1 increases the intracellular levels of reactive oxygen species (ROS) in phagocytic cells (1, 10) as well as nonphagocytic cells (28, 57, 58). Mutants of Rac1 lacking the insert region failed to activate the phagocytic NADPH oxidase complex in vitro (13, 41) and to induce ROS production in REF-52 fibroblasts (24). One of the best-characterized effects of ROS in cells is the activation of the antiapoptotic NF-κB transcription factor (4, 56), and deletion of the insert region was also found to abolish Rac1 activation of NF-κB (24). Thus, the observation that the insert region is also crucial for the antiapoptotic activity of Rac1 in REF-52 cells (25) may be due to the loss of the antiapoptotic function of NF-κB.

In the present study, we have evaluated the importance of the insert region in Rac1 transformation. Surprisingly, in contrast to what has been observed for Cdc42 and RhoA (64, 67), we found that deletion of the insert sequence did not abolish the transforming activity of the constitutively activated Rac1(61L) mutant when assessed in NIH 3T3 cells. Unexpectedly, in contrast to previous observations in REF-52 cells, we also found that the insert sequence was not essential for Rac1 production of ROS and activation of NF-κB in NIH 3T3 cells. However, we observed that deletion of the insert sequence perturbed the ability of Rac1 to activate SRF and to promote the formation of membrane ruffles. Our observations suggest that the insert region of Rac1 is functionally distinct from the insert regions of RhoA and Cdc42 in regulating effector functions involved in the regulation of cellular transformation and actin cytoskeletal organization.

MATERIALS AND METHODS

Molecular constructs. Mammalian expression vectors encoding the constitutively activated and transforming Rac1(61L) mutant [pCGN-*rac*1(61L)] or the NH_2-terminally truncated and transforming Raf-22W mutant (pZIP-*raf*-22W) have been described and characterized previously (62). Reporter plasmids in which luciferase gene expression is regulated by minimal promoter sequences containing responsive elements for SRF, NF-κB, and c-Jun or the human cyclin D1 promoter (pCD1-luc) have been described previously (2, 62). The E2F-1-responsive luciferase reporter containing the luciferase gene under the control of the human E2F-1 promoter was a kind gift from Peggy J. Farnham (Madison, Wis.) and has been described and characterized previously (21).

Rac1(61L) mutagenesis. To assess the role of the insert region in human Rac1 function, we generated mutant cDNA sequences that encoded either single amino acid substitutions (at residues 124, 127, and 130; see Fig. 1) or a deletion of the entire insert region (residues 124 to 135) of the constitutively activated Rac1(61L) mutant protein. The human *rac1*(61L) cDNA sequence was subcloned into the *Bam*HI site of pBluescript SK+ (pBS SK+) and mutagenized using the Chameleon kit (Stratagene). The mutagenic oligonucleotides were generated in an Applied Biosystems synthesizer and were 5′ phosphorylated. The selection primer was used to change the *Xho*I site of pBS SK+ to an *Nde*I site as described by Westwick et al. (62). Sequences of the *rac1* mutagenesis oligonucleotides were as follows: D124A, 5′ CAG TTT CTC GAT CGT TGC TTT ATC ATC CCT AAG 3′; E127A, 5′ CTT CAG TTT TGC GAT CGT GT 3′; and K130A, 5′ CAG CTT CTT CTC TGC CAG TTT CTC G 3′. The insert deletion mutant, designated Rac1(61L, Δins), was designed identically to that described by Freeman et al. (13), in which residues 124 to 135 were deleted and proline 136 was replaced with an alanine, and was constructed as follows. Two restriction enzymes, *Ban*I and *Nco*I, were used to digest the *rac1*(61L) cDNA at positions 305 and 431, respectively. The DNA stretch (base pairs 306 to 431) was replaced by another DNA sequence (lacking base pairs coding for the insert region) formed of two annealed oligonucleotide pairs, A1-C3 and B2-D4. Sequences of the oligonucleotides were as follows, with restriction sites for *Ban*I and *Nco*I underlined: A1, 5′ GCA CCACTG TCC CAA CAC TCC CAT CAT CCT AGT GGG AAC TAA ACT TGA 3′; B2, 5′ TCT TAG GGA TGA TAA AGC AAT CAC CTA TCC GCA GGG TCT AGC 3′; C3, 5′ TTT ATC ATC CCT AAG ATC AAG TTT AGT TCC CAC TAG GAT GAT GGG AGT GTT GGG ACA GTG 3′; and D4, 5′ CAT GGC TAG ACC CTG CGG ATA GGT GAT TGC 3′. Introduction of the desired mutations in the *rac1* cDNA sequences was verified by automated sequencing.

Transient expression reporter analyses. For transient luciferase assays, NIH 3T3 cells were cotransfected with the pCGN-*rac1* expression constructs and pCD1-luc or the SRF-, c-Jun-, NF-κB-, or E2F-1-responsive luciferase reporter plasmid using the calcium phosphate precipitation method (5) and were grown in Dulbecco's modified Eagle medium supplemented with 10% calf serum for 48 h. Cultures were subsequently starved for 14 to 18 h in medium supplemented with 0.5% calf serum. The cells were then lysed, and luciferase activity was measured using enhanced chemiluminescence reagents (Amersham) in a Monolight 2010 luminometer (Analytical Luminescence, San Diego, Calif.).

NIH 3T3 cell transformation assays. The consequences of the insert sequence mutations on Rac1(61L) transforming activity were determined using two different assays. First, the ability of Rac1(61L) mutants to cooperate with activated Raf-22W to cause synergistic enhancement of focus-forming activity in NIH 3T3 cells was assayed as described previously (62). Briefly, subconfluent cultures of NIH 3T3 cells seeded in 60-mm-diameter tissue culture dishes were cotransfected with 500 ng of pCGN-*rac1*(61L) plasmid DNA together with either 50 ng of pZIP-*raf*-22W or empty pZIP-NeoSV(x)1 plasmid DNA. The cultures were replenished with fresh growth medium on alternate days until foci of transformed cells were detected 14 to 16 days later. Cells were fixed in methanol-acetic acid (10% each) solution and stained with 0.4% crystal violet, and the number of foci was quantitated under the microscope. Second, NIH 3T3 cells stably expressing each Rac1 protein were analyzed for the ability to proliferate in an anchorage-independent environment in soft agar. NIH 3T3 cells transfected with 500 ng of the various Rac1 mutant constructs were selected in growth medium supplemented with hygromycin (200 μg per ml) for 7 to 10 days. Multiple drug-resistant colonies were then pooled together, and the level of exogenous Rac1 protein expression in each cell population was determined by Western blot analysis using antihemagglutinin (anti-HA) epitope antibody (BAbCO). Single-cell suspensions (10^4 cells per 60-mm dish) of each cell population were then plated in growth medium supplemented with 0.3% agar (5). The appearance of colonies of proliferating cells was monitored for up to 18 days.

Measurement of cellular proliferation. Rac1 stimulates cell cycle progression, causing an increase in DNA synthesis and cellular proliferation (43). To analyze the role of the insert region in Rac1-induced proliferation, we assessed the ability of Rac1(61L, Δins) to drive DNA synthesis in NIH 3T3 cells using a colorimetric cell proliferation enzyme-linked immunosorbent assay (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, NIH 3T3 cells stably expressing Rac1(61L) or Rac1(61L, Δins) or the cognate pCGN vector were cultured in 96-well plates at 30×10^3 cells/well and serum starved for 24 h in growth medium supplemented with 0.5% calf serum. The culture medium was subsequently replenished with growth medium supplemented with 0.5% calf serum and with a 10 μM final concentration of the thymidine analogue 5-bromo-2′-deoxyuridine (BrdU) for 28 h (labeling medium). The labeling medium was then removed and cells were fixed prior to DNA denaturation. Measurement of BrdU incorporation into DNA was assessed photometrically in a Benchmark microplate reader (Bio-Rad) at a 655-nm absorbance wavelength.

Measurement of ROS production. Rac1 elevates cellular levels of ROS in many cell types, including fibroblasts. To assess the involvement of the insert region in Rac1-mediated ROS production, we measured intracellular levels of ROS using the peroxide-sensitive and fluorescent dye 2′,7′-dichlorohydrofluorescein diacetate (H_2DCFDA; Molecular Probes, Eugene, Oreg.) (56, 58). NIH 3T3 cells stably expressing each Rac1(61L) protein or an empty vector-transfected (control) cell population was plated at equal density on glass coverslips

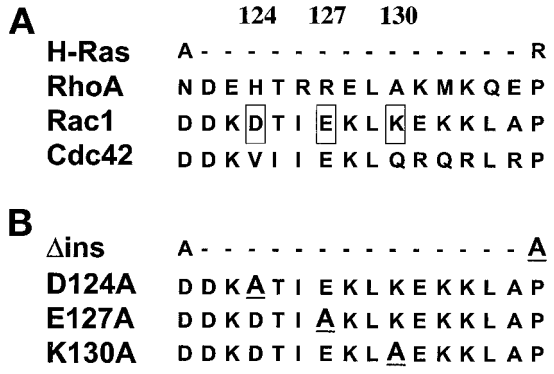

FIG. 1. Mutation of the Rac1 insert region. (A) Alignment of the insert sequences of RhoA, Rac1, and Cdc42 with that of H-Ras. (B) Sequences of the Rac1 mutants showing altered residues in bold and underlined. The Rac1(61L, Δins) mutant lacks amino acids 124 to 135, with proline 136 mutated to alanine.

and maintained in growth medium for 24 h. The complete medium was subsequently replaced by growth medium supplemented with 0.5% calf serum for 14 h. Coverslips were then washed twice in Hank's buffered saline solution (HBSS; Gibco-BRL) lacking phenol red and incubated in 10 μM fresh H_2DCFDA (Molecular Probes) in Hank's buffered saline solution for 15 min at room temperature (4, 58). Images were immediately collected on a laser confocal microscope (Leica TCS-NT) using a long pass of a 515-nm filter.

Immunofluorescence analyses. The expression and localization of the different Rac1 mutants were analyzed by fluorescence microscopy. NIH 3T3 cells stably expressing Rac1 proteins were plated on glass coverslips, maintained in growth medium for 6 h, and serum starved (0.5%) for 14 to 18 h. The cells were then fixed in 3.6% formaldehyde–phosphate-buffered saline solution, blocked in 3% bovine serum albumin, and permeabilized by treatment with 0.1% Triton X-100. Cultures were probed first with anti-HA monoclonal antibody (MAb) (BAbCO) followed by rhodamine-conjugated anti-mouse immunoglobulin G (Jackson ImmunoResearch Labs, West Grove, Pa.). Cytoskeletal actin organization was analyzed by detecting F-actin distribution using fluorescein isothiocyanate (FITC)-conjugated phalloidin (Jackson Labs). Cells were photographed at a ×100 magnification using a Zeiss Axiophot fluorescence microscope.

RESULTS

To evaluate the contribution of the Rho insert region to Rac1 signaling and transformation, we introduced either missense or deletion mutations into the insert region of the constitutively activated and transforming Rac1(61L) mutant (Fig. 1). First, we introduced single amino acid substitutions (D124A, E127A, and K130A) at insert region residues that are solvent accessible and may facilitate Rac1 interaction with other proteins (20). We introduced alanine substitutions to minimize the perturbation of the helical nature of the insert region. Second, we also created a more extensive mutation by the deletion of residues 124 to 135 and the conversion of proline 136 to alanine. The resulting mutant was designated Rac1(61L, Δins) and is identical to the one utilized in previous studies using the constitutively activated Rac1(12V) protein (13, 24, 25).

The insert region is not required for Rac1 transforming activity. Previous studies established the importance of an intact insert region for Cdc42 and RhoA transforming activities in NIH 3T3 cells (64, 67). Similarly, an intact insert region was also found to be required for Rac1-stimulated mitogenesis in transient expression analyses in REF-52 cells (24). However, whether the insert is important for Rac1 transformation has not been determined. To address this question, we have assessed the contribution of this region to Rac1-mediated transformation of NIH 3T3 cells using focus formation and soft agar analyses. We and others have shown previously that Rac1 cooperates with activated Raf in focus formation transformation assays (29, 46). As shown in Fig. 2, cultures transfected with plasmids encoding either activated Rac1(61L) or activated Raf-22W alone caused little or no focus-forming activity. However, cotransfection of both plasmids caused a synergistic focus-forming activity of over 80 foci per dish. Surprisingly, the insert point mutants as well as the deletion mutant of Rac1(61L) all retained strong cooperative focus-forming activity when coexpressed with Raf-22W. Interestingly, we found that Rac1(61L, Δins) showed a reproducible ~20% greater focus formation activity than nonmutated Rac1(61L). Additionally, Rac1(61L, Δins)-induced foci were detectable at an earlier time (8 days after transfection) and generally progressed to larger sizes than those caused by Rac1(61L). Thus, the deletion of the insert region caused a modest but reproducibly observed increase rather than a decrease in Rac1(61L) transforming activity.

Next, we determined if an intact insert region was also dispensable for Rac1(61L) induction of anchorage-independent growth when cells are suspended in soft agar. For these analyses, mass populations of NIH 3T3 cells were stably transfected with the empty pCGN-hygro expression plasmid (vector) or pCGN-hygro plasmid constructs encoding nonmutated or insert region-mutated versions of Rac1(61L). Western blot analyses showed that the established cell lines had comparable steady-state levels of expression of Rac1(61L) and each insert region mutant protein, indicating that the mutations did not cause a significant change in protein stability (Fig. 3). Whereas empty vector-transfected NIH 3T3 cells did not form colonies when suspended in soft agar (Fig. 4), cells stably expressing the Rac1(61L) protein were able to proliferate under anchorage-independent conditions. Again, the insert mutants retained their transforming potential, with robust formation of multicellular colonies after 18 days in culture. It is noteworthy that, similar to what was seen with the focus formation assays, Rac1(61L, Δins)-expressing cells displayed an enhanced colony-forming activity with a higher frequency and an earlier onset of colony formation.

Furthermore, we assessed the role of the insert region in Rac1-induced cell cycle progression by measuring the ability of Rac1(61L) and Rac1(61L, Δins) to promote mitogenesis in NIH 3T3 cells (Fig. 5). Pooled populations of unsynchronized serum-starved cells stably expressing empty vector, Rac1(61L), or Rac1(61L, Δins) were incubated in the presence of the thymidine analogue BrdU. Progression of cells through the S phase of the cell cycle was probed by quantitating the incorporation of BrdU into DNA. As shown in Fig. 5, Rac1(61L) caused about a 50% increase in BrdU incorporation relative to the levels seen in vector-expressing cells. Surprisingly, deletion of the insert region did not inhibit the mitogenic ability of Rac1 in NIH 3T3 cells, as Rac1(61L, Δins) caused an increase in BrdU incorporation (1.87-fold) to levels that reproducibly exceeded those seen with Rac1(61L)-expressing cells. Taken together, these data exclude the involvement of the insert region of Rac1 in growth and transformation and contrast with the critical role of the insert sequences in mediating Cdc42 and RhoA transforming activity when assessed in NIH 3T3 cells.

An intact insert region is required for Rac1 induction of SRF but not for cyclin D1 promoter-, c-Jun-, or E2F-1-medi-

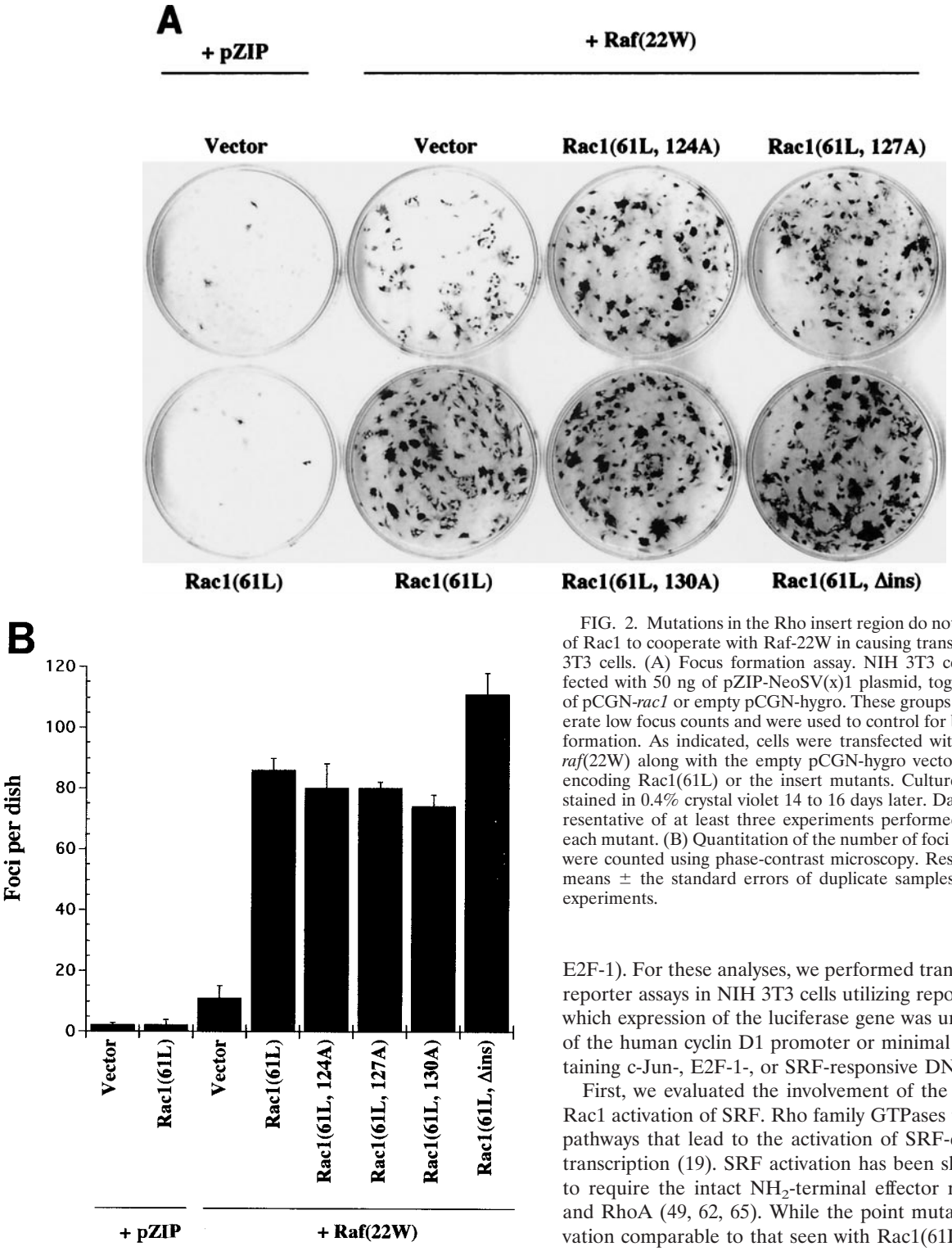

FIG. 2. Mutations in the Rho insert region do not impair the ability of Rac1 to cooperate with Raf-22W in causing transformation in NIH 3T3 cells. (A) Focus formation assay. NIH 3T3 cells were cotransfected with 50 ng of pZIP-NeoSV(x)1 plasmid, together with 500 ng of pCGN-*rac1* or empty pCGN-hygro. These groups reproducibly generate low focus counts and were used to control for background transformation. As indicated, cells were transfected with 50 ng of pZIP-*raf*(22W) along with the empty pCGN-hygro vector or pCGN-hygro encoding Rac1(61L) or the insert mutants. Cultures were fixed and stained in 0.4% crystal violet 14 to 16 days later. Data shown are representative of at least three experiments performed in duplicate for each mutant. (B) Quantitation of the number of foci per dish. Colonies were counted using phase-contrast microscopy. Results represent the means ± the standard errors of duplicate samples in at least three experiments.

ated transcription. The growth promoting activity of Rac1 may be mediated in part by regulating gene expression or cell cycle progression. Therefore, we assessed whether the insert region was involved in Rac1 activation of transcription factors that regulate the expression of growth factor-responsive genes (SRF and Jun) or the function of components important in the regulation of the G_1 phase of the cell cycle (cyclin D1 and E2F-1). For these analyses, we performed transient expression reporter assays in NIH 3T3 cells utilizing reporter plasmids in which expression of the luciferase gene was under the control of the human cyclin D1 promoter or minimal promoters containing c-Jun-, E2F-1-, or SRF-responsive DNA elements.

First, we evaluated the involvement of the insert region in Rac1 activation of SRF. Rho family GTPases trigger signaling pathways that lead to the activation of SRF-dependent gene transcription (19). SRF activation has been shown previously to require the intact NH_2-terminal effector regions of Rac1 and RhoA (49, 62, 65). While the point mutants caused activation comparable to that seen with Rac1(61L) (20-fold), the deletion mutant showed a near-complete loss in its ability to stimulate SRF (80% reduction) (Fig. 6A). Thus, an intact insert region, as well as the core effector domain, is important in mediating Rac1 stimulation of SRF activity.

Rac1 is an activator of the JNK mitogen-activated protein kinase pathway. Activated JNK causes phosphorylation and activation of the c-Jun transcription factor, which is a component of the AP-1 transcription factor and a key regulator of

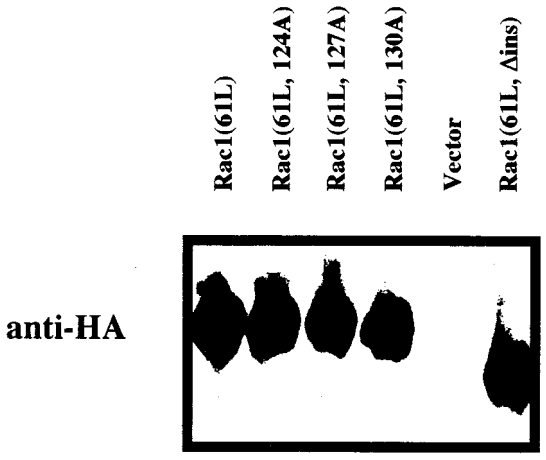

FIG. 3. Mutations of the Rho insert region do not perturb protein stability. Hygromycin-selected NIH 3T3 cells stably expressing HA epitope-tagged Rac1(61L) or the indicated insert mutant protein were lysed. The levels of Rac1 proteins expressed in each lysate were determined by Western blotting using anti-HA MAbs (BAbCO). Comparable levels of expression were detected in stable transfections of NIH 3T3 cells or in transient overexpression analyses in COS-7 cells (data not shown).

early response and growth-promoting genes (6, 8, 37). We determined previously that Rac1 regulates SRF and Jun activation via distinct effector pathways (62). Therefore, we determined if an intact insert region was required for Rac1 activation of c-Jun. Transient expression of Rac1(61L) caused a sixfold stimulation of transcription from a c-Jun-responsive luciferase promoter plasmid (Fig. 6B). Comparable stimulation was seen with the missense or deletion mutants of Rac1(61L), indicating that the insert region is dispensable for Rac1 activation of the JNK–c-Jun pathway.

Rac1 has a crucial function in the transition of cells through the G_1 phase of the cell cycle and is needed for Ras- and growth factor-induced DNA synthesis (43). Furthermore, both Ras and Rac control cell cycle progression, in part by stimulating cyclin D1 expression (2, 62). We therefore tested whether mutations in the insert region affected the ability of Rac1(61L) to stimulate transcription from the cyclin D1 promoter. We found that the point mutants as well as the deletion mutant stimulated transcription from the cyclin D1 promoter to levels that were comparable to that seen with their nonmutated counterpart (Fig. 6C). These results indicate that Rac1-mediated cyclin D1 activation does not require the insert region.

Rac1 may alter cell cycle progression by activation of E2F transcription factors, which in turn stimulate the expression of genes important for transition through the G_1 phase of the cell cycle (17). To address the importance of the insert region in Rac1 regulation of E2F-1-responsive gene expression, we performed transient expression analyses in NIH 3T3 cells using a reporter plasmid in which the luciferase gene was regulated by E2F-1-responsive promoter elements. Rac1(61L), Rac1(61L, 124A), and Rac1(61L, Δins) caused a ninefold induction in luciferase activity (Fig. 6D). The Rac1(61L, 127A) and Rac1 (61L, 130A) insert mutants showed limited (20 to 30%) reductions in stimulation. However, since the deletion mutant showed no reduction in activity, we concluded that the insert of Rac1 is not critical for Rac1 activation of E2F-1-dependent gene expression and that the enhanced transforming potency of Rac1(61L, Δins) did not correlate with an enhanced ability to activate these signaling pathways.

An intact insert region is dispensable for Rac1 activation of NF-κB-mediated transcription and production of ROS. Rho family proteins and their GEFs promote NF-κB translocation to the nucleus with subsequent stimulation of NF-κB-mediated transcriptional activation (39, 44, 56). Previous microinjection analyses showed that deletion of the insert region impaired the ability of Rac1(61L) to activate NF-κB in REF-52 rat fibroblast cells (25). Therefore, we evaluated whether the point mutations or the deletion also impaired the ability of Rac1(61L) to stimulate NF-κB-dependent transcription in NIH 3T3 cells. As shown in Fig. 7, Rac1(61L) caused a fivefold enhancement of NF-κB-dependent transcription in transiently transfected NIH 3T3 cells. Surprisingly, all the mutants of the insert region retained strong abilities to stimulate transcription. In particular, Rac1(61L, Δins) showed an enhanced (~50%) ability to stimulate NF-κB-mediated transcription. These results show clearly that the insert region of Rac1 is not required for NF-κB transcriptional upregulation.

Previous studies indicated that Rac1 superoxide production is linked to Rac1 activation of NF-κB (4, 56). Furthermore, the insert region was found to be required for Rac1 stimulation of superoxide production when assayed by microinjection analyses in COS-1 cells (24). Since we found that the insert region was not required for NF-κB activation, we determined whether our mutants retained the ability to stimulate ROS production. To this end, we have used the peroxide-sensitive marker DCFDA to compare the levels of ROS production in NIH 3T3 cells stably expressing Rac1(61L, Δins) versus those expressing Rac1(61L). This compound has been used successfully to detect growth factor-, Ras-, and Rac1-dependent upregulation of ROS levels in NIH 3T3 cells (58) as well as other cell types (33, 42, 53, 56, 57). NIH 3T3 cells stably expressing Rac1(61L) exhibited a four- to fivefold enhancement in their ROS levels above those seen in the empty vector-transfected control cells (Fig. 8). Surprisingly, we found that cells stably expressing Rac1 (61L, Δins) displayed a twofold higher ROS content relative to that of Rac1(61L)-expressing cells. The increased ROS production was not due to nonspecific protein expression, as Rac1(N17)-expressing cells exhibited no increase in DCFDA fluorescence (data not shown). Thus, consistent with our NF-κB observation, we found that the insert region of Rac1 is also not required for Rac1-induced production of ROS in NIH 3T3 cells.

An intact insert region is required for Rac1-induced membrane ruffling. Rac1 activation stimulates the reorganization of cytoskeletal actin, leading to the formation of lamellipodia and membrane ruffles (48). We therefore determined if mutation or deletion of the insert region disrupted the ability of Rac1 to cause membrane ruffling in NIH 3T3 cells. For these studies, NIH 3T3 cells stably expressing Rac1(61L) or Rac1(61L, Δins) were fixed and probed with anti-HA epitope antibody coupled to rhodamine to detect HA-Rac1 expression and with FITC-conjugated phalloidin to visualize F-actin. Expression of Rac1 (61L) (Fig. 9C) disrupted basal stress fiber formation (Fig. 9B) and caused a redistribution of actin to membrane structures, forming lamellipodia or membrane ruffles (Fig. 9D). Expression of the Rac1(61L, Δins) mutant (Fig. 9E) induced relocalization of actin from stress fibers to the cell membrane, but no

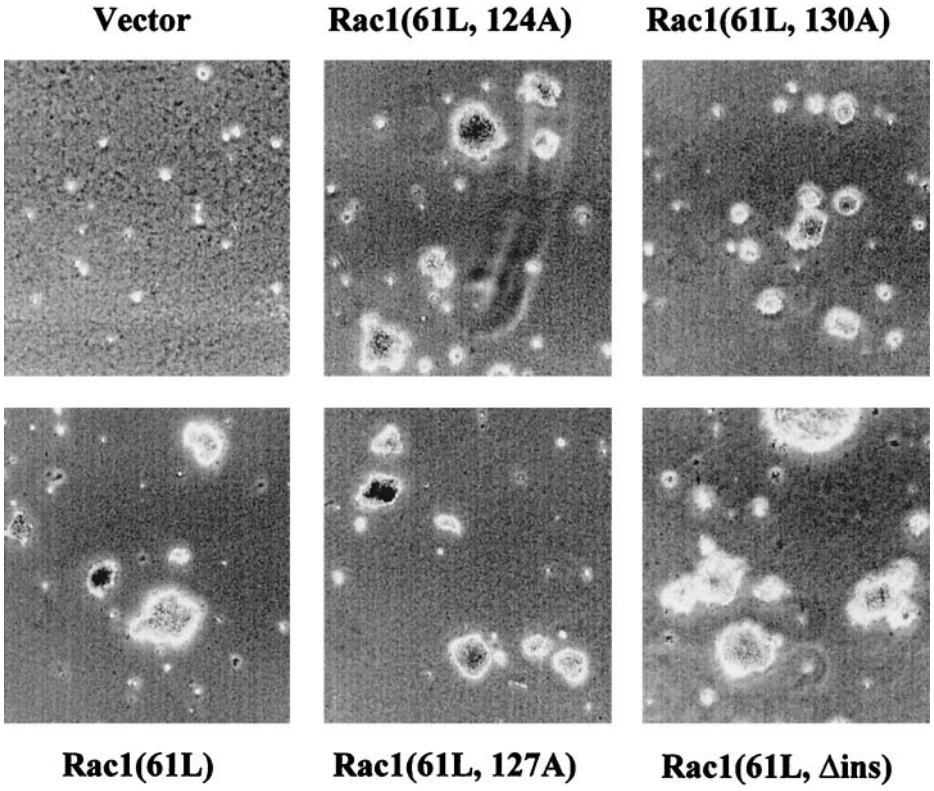

FIG. 4. The insert is not involved in Rac1-mediated anchorage-independent growth of NIH 3T3 cells in soft agar. Pooled populations of NIH 3T3 cells stably transfected with the empty pCGN-hygro vector or vector encoding the indicated Rac1 mutant were assayed for the ability to grow under anchorage-independent conditions. Cells (2×10^4) of the indicated groups were seeded into growth medium supplemented with 0.3% agar, and colonies were photographed 18 days later under ×40 magnification. Results are representative of two independent experiments performed in triplicates.

membrane ruffling activity was detected (Fig. 9F). Interestingly, Rac1(61L, Δins)-expressing cells displayed actin-rich microspikes at the plasma membrane, an activity more characteristically associated with the activation of Cdc42. This phenotype was independent of any secondary genetic alterations that may have occurred during cell passage, since we observed similar results when the analyses were done on transiently transfected NIH 3T3 cells (data not shown). Moreover, the same phenotype was seen using independently generated clones of the *rac1*(61L, Δins) cDNA. These findings suggest that the insert of Rac1 is required for membrane ruffling activity and that deletion of this region may cause Rac1 to promote actin reorganization changes similar to those caused by activated Cdc42. These results contrast with those seen with Cdc42 and RhoA, where deletion of their insert regions did not perturb their ability to promote filopodia and stress fibers, respectively (64, 67).

DISCUSSION

Members of the Rho family of GTPases share an additional amino acid sequence, termed the Rho insert region, which distinguishes them from all other members of the Ras superfamily. Recent mutational analyses showed that an intact Rho insert was required for the transforming, but not actin reorganization, activities of Cdc42 and RhoA in NIH 3T3 cells (64, 67). In the present study, we assessed the importance of the insert region in Rac1 signaling and transforming activities. Surprisingly, we found that Rac1 transformation of NIH 3T3 cells did not require the insert region. However, we did find that deletion of this region impaired Rac1 activation of SRF and induction of membrane ruffling. Our results emphasize the distinct functional role of the insert region in Rac1 when compared to that of Cdc42 and RhoA.

Our observation that deletion of the insert region did not abolish Rac1 mitogenic and transforming activities contrasts with previous observations that the same deletion caused impairment of the mitogenic function of Rac1 (24). Several differences between the two studies may account for the different conclusions. First, although the same deletion was utilized in both studies, our mutation was introduced into the Rac1(61L) mutant instead of the Rac1(12V) variant utilized by Bar-Sagi and colleagues (24, 25). Although both are constitutively activated and transforming mutants of Rac1, there is evidence that they differ in their affinity for effector binding (32). Hence, it is possible that the insert deletion caused differential impairment of effector interactions in the G12V and the Q61L mutant versions of Rac1. Second, whereas the previous study evaluated the ability of transiently expressed protein to induce DNA synthesis in REF-52 rat fibroblasts, our analyses determined the consequences of sustained Rac1 activation in NIH 3T3 mouse fibroblasts. Support for the potential importance of these differences has been observed in related studies with Ras. Although transient expression of activated Ras is mitogenic in

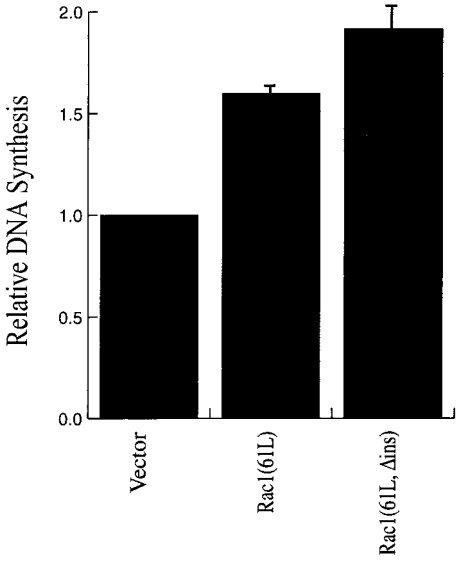

FIG. 5. The insert is not required for Rac1-induced DNA synthesis in NIH 3T3 fibroblasts. DNA synthesis was assessed in pooled populations of asynchronous NIH 3T3 cells stably transfected with empty pCGN-hygro vector or expressing the indicated Rac1 mutant. Serum-starved cells were incubated with a 10 μM final concentration of the thymidine analogue BrdU. Measurement of BrdU incorporation into DNA was assessed photometrically at a 655-nm absorbance wavelength. Data are expressed as relative DNA synthesis over that obtained with empty vector cells. Results are means ± standard errors of four independent experiments performed in octuplets for each indicated group.

REF-52 cells, sustained Ras activation causes cell cycle arrest and senescence in these cells (52). In contrast, transient or sustained activation of Ras causes growth and proliferative responses in NIH 3T3 cells (30). Thus, cell type differences as well as different consequences of transient versus sustained Rac1 activation may also account for the different observations seen in the two studies. Finally, we have determined that the insert region is dispensable for the ability of Rac1 to induce focus formation (Fig. 2), promote soft agar growth (Fig. 4), enhance DNA synthesis (Fig. 5), increase saturation density, and promote growth in low serum (data not shown). These transformation assays, using sustained and transient expression analyses in NIH 3T3 cells, were similar to those used by Wu et al. (64) and Zong et al. (67). Consequently, a comparison of our observations is more straightforward with the studies on Cdc42 and RhoA than with the previous Rac1 studies and establishes the distinct role the insert region plays in the actin-reorganizing and growth-regulating functions of these Rho GTPases.

The involvement of the insert region of Rac1 in ROS production has been derived mainly from in vitro biochemical analyses of the phagocytic NADPH oxidase system (10, 13, 16, 41, 45), despite the fact that other studies reached different conclusions (11, 31, 59). Nevertheless, a recent report demonstrated that truncation of the insert region severely impaired the ability of transiently expressed Rac1 to promote ROS generation in nonphagocytic COS-1 cells (24). This mutant also lacked the ability to trigger NF-κB-mediated survival pathways in REF-52 cells (25), which is consistent with previous observations that implicated ROS production in mediating Rac1 activation of the NF-κB transcription factor (4, 23). In contrast, we show here that NIH 3T3 fibroblasts stably expressing the activated Rac1 (61L) protein stimulated ROS production and NF-κB activation that was not dependent on an intact insert region. Interestingly, we have observed that blocking NF-κB activation in Rac1(61L)-transformed NIH 3T3 cells did not inhibit ROS production but did impair anchorage-independent growth (data not shown), indicating that ROS-mediated activation of NF-κB contributes to Rac1 growth stimulation.

As discussed above, these differences may be due to the differences in activating mutation, cell type, and/or temporal expression. Different cell types possess varying mechanisms for regulating and coping with ROS elevation, and alterations in those levels can trigger growth-promoting or inhibitory signals depending on the origin of the cell system (25, 26, 33). There is evidence that ROS-dependent NF-κB activation is cell type specific (4), and it would not be surprising if ROS thresholds required to activate NF-κB are also cell type dependent and isoform specific (7). The recent description of multiple tissue-specific gp91 phox homologues such as Mox1 and Renox (15, 55) suggests that multiple genes code for distinct NADPH oxidase-like systems. Consequently, these observations raise the possibility that some NADPH oxidase systems particular to NIH 3T3 but not REF-52 cells may not require the insert region of Rac1, while others may require it. Further analyses characterizing the mechanisms of Rac1-mediated ROS generation in fibroblasts will be required before we can determine the basis for the observed differences.

One significant consequence of the insert region deletion was its abrogation of the ability of Rac1 to promote membrane ruffling. In contrast to a previous transient expression analysis in COS-1 cells (24), we observed that transient or stable expression of the insert region deletion mutant in NIH 3T3 cells reproducibly induced the dissolution of actin stress fibers together with the appearance of filopodium-like actin structures at the cell periphery. Differences in the cytoskeletal phenotypes induced by Rac1 effector domain mutants [for example, Rac1 (37A)] have been observed in different cell types (50, 62), and it would not be surprising that COS-1 and NIH 3T3 cells also exhibit similar variations. Finally, the observation that the insert region was not involved in Cdc42-mediated actin changes in NIH 3T3 cells (64) while the insert sequence was essential for Rac1-mediated membrane ruffling in the same cell system further emphasizes the distinct role the insert region plays in different Rho GTPases.

Several possible scenarios may explain our observations of the Rac1 (61L, Δins) actin reorganization activity. First, deletion of the insert region may have impaired Rac1 interaction with an effector(s) important for membrane ruffling regulation. Second, the deletion of the insert region may have caused a dominant-negative phenotype for Rac1. For example, it was shown that a dominant-negative mutant of one Rho family protein may actually promote actin reorganization changes caused by a different Rho family member (40). Accordingly, the insert region deletion mutant may act as a dominant-negative inhibitor of membrane ruffling, thereby shifting the balance of activity of Rac1 relative to other Rho family members, resulting in the induction of filopodium-like actin projections.

In addition to the impairment in membrane ruffling, the insert mutant was also impaired in its ability to activate SRF.

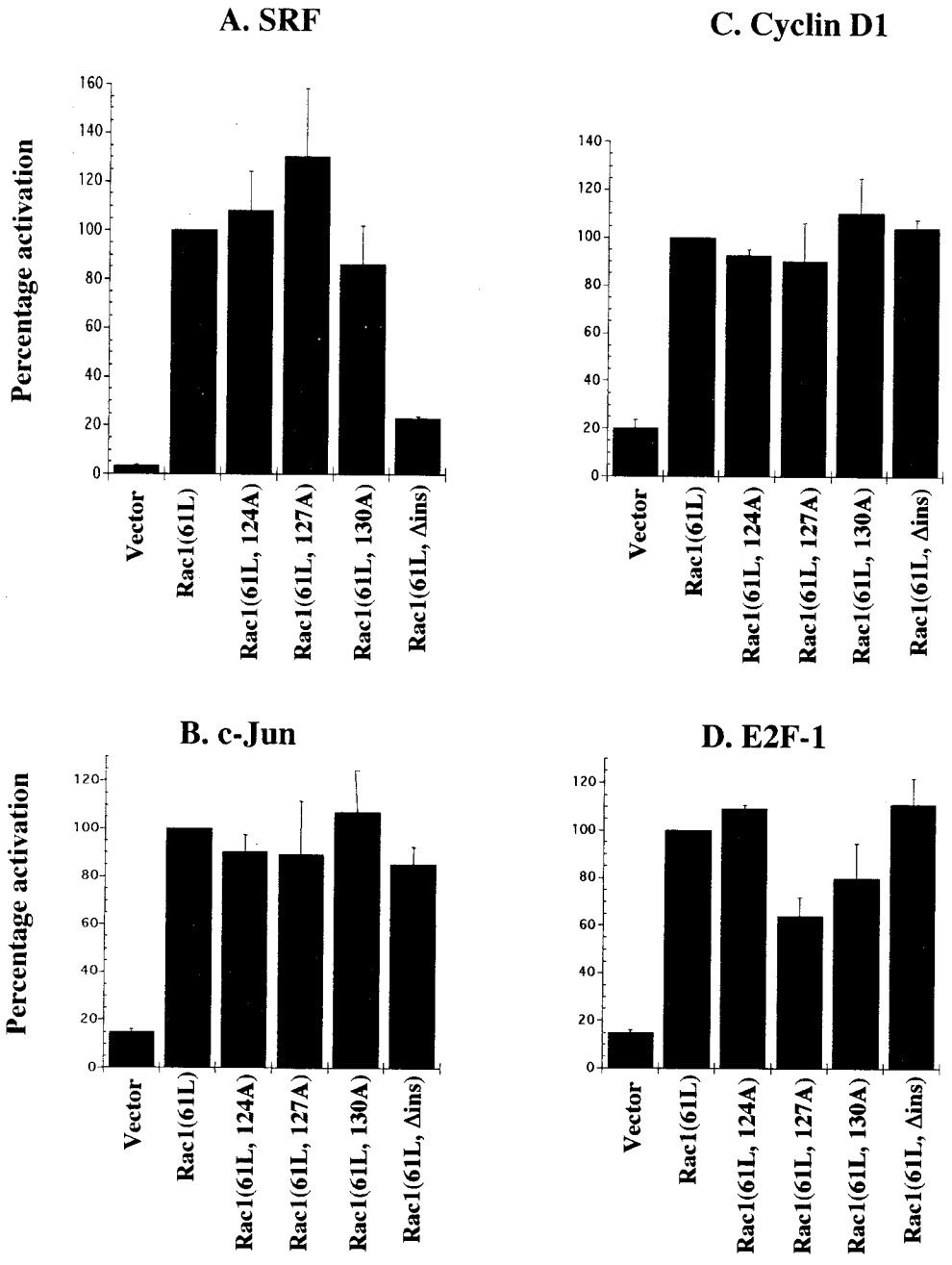

FIG. 6. The insert region is required for Rac1 activation of SRF, but not cyclin D1, c-Jun, or E2F-1, in NIH 3T3 cells. Cells were transiently transfected with 1.5 μg of luciferase reporter constructs encoding promoter elements for SRF (A), c-Jun (B), cyclin D1 (C), or E2F-1 (D), along with 1 μg of cognate pCGN vector or the indicated Rac1 mutants using the calcium phosphate precipitation method. Forty-eight hours later, cells were starved in medium supplemented with 0.5% calf serum for another 14 to 18 h and then lysed. Cell extracts were analyzed using enhanced chemiluminescence reagents (Amersham). Results are expressed as percent activation relative to that observed with Rac1(61L). Average luciferase activity above that of the vector control seen with Rac1(61L) was 28.6-fold for SRF, 5-fold for cyclin D1, 6.7-fold for c-Jun, and 6.67-fold for E2F-1. Data shown represent means ± standard errors of at least three independent experiments performed in duplicate for each group. All proteins were expressed to similar levels (data not shown).

Several observations suggest that there may be a connection between SRF activation and cytoskeletal remodeling. Treisman and coworkers have shown that SRF activation is responsive to the relative levels of monomeric G and polymerized F actin (54). Similarly, a Rac1(37A) mutant unable to activate SRF (62) was found to be aberrant in membrane-ruffling activity while promoting filopodium formation in NIH 3T3 cells (50). This SRF inactivation paralleled repression of membrane ruffling with a concomitant induction of filopodium-like structures in our system using the Rac1(61L, Δins) mutant. Whether this correlation holds true for other mutants of Rac1 merits further investigation.

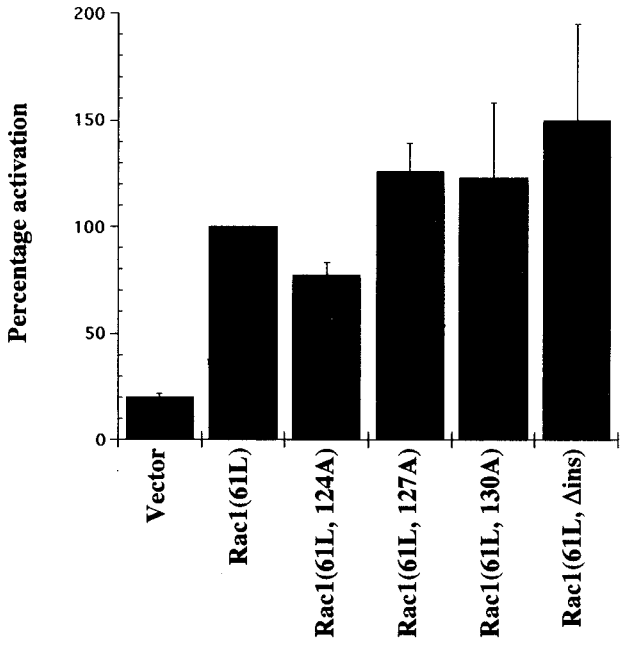

FIG. 7. The insert region is dispensable for Rac1 activation of NF-κB-dependent transcriptional activation in NIH 3T3 cells. Cells were cotransfected with 1.5 μg of NF-κB luciferase reporter plasmid and 1 μg of the indicated Rac1 mutant or vector control. Results were analyzed and expressed as for Fig. 5. Data shown represent the averages of three independent assays performed in duplicates (± standard errors).

Interestingly, subtle point mutations in the insert region (124A, 127A, and 130A), in contrast to its entire deletion, did not affect the ability of Rac1(61L) to cause membrane ruffling (data not shown) and to stimulate SRF activity in NIH 3T3 cells. One possible interpretation of these results is that the insert deletion induced alterations distal to the mutation site, which then resulted in the changes seen in SRF and membrane ruffling. However, our recent nuclear magnetic resonance analyses of the Rac1(Δins) mutant, when compared to the wild-type Rac1 protein, revealed no major structural perturbations upon deletion of the insert sequence (R. Thapar, A. E. Karnoub, and S. L. Campbell, unpublished data). This observation supports the model that the insert is involved in direct interactions with an effector(s) controlling actin reorganization and/or SRF activation.

The fact that the insert region may serve a distinct role in different Rho family GTPases is not unexpected considering that the insert region is one of the sequences of greatest divergence among Rho family proteins (60). This divergence extends beyond sequence homology. First, the Rho insert region assumes different structural conformations in different Rho family GTPases. The crystal structures of Rac1 (20) and RhoA (22) showed that their inserts each form two short α-helices followed by an extended loop. However, the nuclear magnetic resonance solution structure of Cdc42 shows no evidence for one of the helices and the other lies in a region of low electron density (12). Additionally, the insert of Cdc42Hs is very flexible and lies in close proximity to residues 84 to 89 (12, 35), while the insert of Rac1 was shown to be mostly solvent exposed (20).

Second, the insert region mediates differential interactions with various partners. For instance, in Cdc42 the insert is important for guanine nucleotide dissociation inhibitor-mediated GDP release (63) and forms a binding interface for IQGAP1 but not for PAK1 or WASP (34). Similarly, Missy et al. showed that the insert of Rac1 but not of Cdc42 is involved in binding phosphoinositide 3,4,5-triphosphate and phosphoinositide 4,5-biphosphate (38). Together these results suggest that the Rho insert region, by (i) having different sequence and structural features, (ii) mediating different biochemical interactions, and (iii) assuming different biological functions, may act as a specificity-determining regulatory unit conducting distinct effector interactions in various Rho family GTPases.

In summary, our results do not implicate the insert region of Rac1 in cellular transformation or superoxide production in NIH 3T3 cells. Instead, we show that this region is crucial for

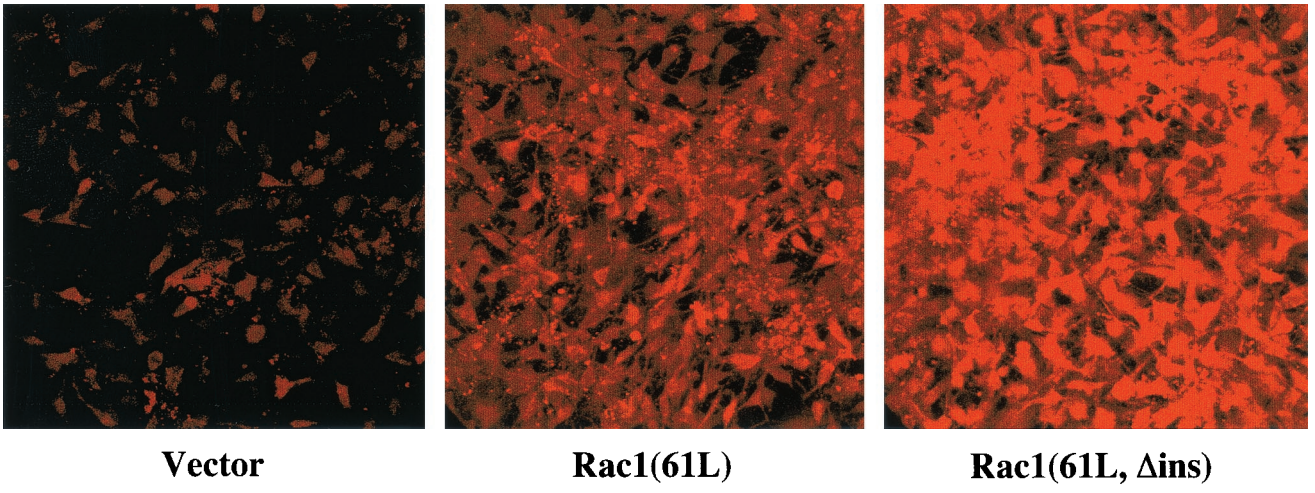

FIG. 8. Deletion of the insert region potentiates Rac1-induced production of ROS. NIH 3T3 cells stably transfected with the empty pCGN-hygro vector or pCGN-hygro encoding Rac(61L) or Rac1(61L, Δins) were plated on glass coverslips and starved for 14 h. Cells were then washed and incubated in 10 μM DCFDA dye for 15 min at room temperature. Slides were scanned immediately by laser confocal microscopy using a >515-nm filter. Results shown are representatives of three independent experiments.

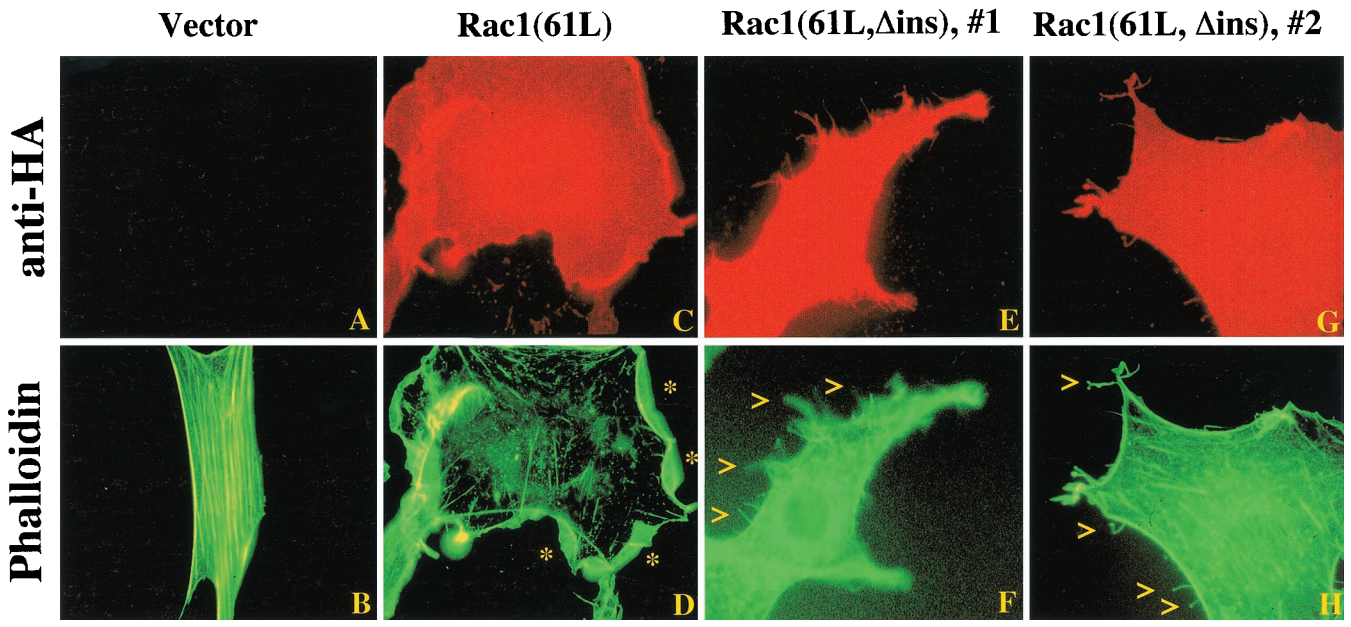

FIG. 9. The insert region controls Rac1 membrane ruffling activity in NIH 3T3 cells. NIH 3T3 stable cell lines of the indicated groups were plated on coverslips, starved, fixed in 3.6% formaldehyde–phosphate-buffered saline solution, and permeabilized with 3% bovine serum albumin–0.1% Triton X-100–phosphate-buffered saline solution. Exogenously expressed HA-tagged Rac1(61L) or Rac1(61L, Δins) protein was probed with anti-HA epitope MAbs (BAbCO; 1:500 dilution). F-actin distribution was detected with FITC-conjugated phalloidin (Jackson Labs; 1:1,000 dilution). Immunostaining was captured with a Leica Axiophot fluorescent microscope, and photographs were taken with a ×100 oil lens. Results are representative of at least five independently generated cell lines of each group. Results were similar using two independently generated clones of the Rac1(61L, Δins) mutant. The same observations were seen in analyses of transiently transfected NIH 3T3 cells (data not shown). *, lamellipodia; >, filopodium-like extensions.

Rac1 induction of membrane ruffling and SRF activation. These data suggest that the Rho insert region, unlike the core effector domain, does not serve a common function in all Rho family GTPases. Future studies to investigate what role the Rac1 insert region plays in effector recognition and whether it interacts with unique effector targets will help to clarify the importance of the insert region in Rac1 function.

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